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Soluble and Microsomal Glutathione S-Transferase Activities in Pea Seedlings (*Pisum sativum* L.)

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Abstract. Epicotyl and primary leaves of pea seedlings (*Pisum sativum* L., var. Alaska) were found to contain soluble and microsomal enzymes catalyzing the addition of glutathione to the olefinic double bond of cinnamic acid. Glutathione S-cinnamoyl transfer was also obtained with enzyme preparations from potato slices and cell suspension cultures of parsley and soybean.

The pea transferases had pH-optima between pH 7.4 and 7.8 K_m-values were 0.1–0.4 mM and 1–4 mM for cinnamic acid and glutathione, respectively. V-values were between 2–15 nmol mg^{-1} protein × min.

Chromatography on Sephacryl S-200 indicated that the soluble pea glutathione S-cinnamoyl transferase activity existed in molecular weight forms of 37,000, 75,000, and 150,000. The glutathione-dependent cleavage of the herbicide fluorodifen was catalyzed by a different soluble enzyme activity which eluted in molecular weight positions of 47,000 and/or 82,000.

The microsomal fraction from pea primary leaves also catalyzed the conjugation of the carcinogen benzo[α]pyrene with glutathione.

Key words: Cinnamic acid – Fluorodifen – Glutathione – S-transferases – *Pisum*.

Introduction

Glutathione (GSH) S-transferase activities constitute an important group of the detoxifying enzyme systems of mammalian liver (Boyland and Chasseaud, 1969; Jakoby, 1978). Several transferase activities for the conjugation of herbicides with GSH have also been discovered in plants (Shimabukuro et al., 1978).

Abbreviations: GSH = glutathione; DDE = 1,1-Dichloro-2,2-bis-(4-chlorophenyl)-ethylene; DDMU = 1-Chloro-2,2-bis-(4-chlorophenyl)-ethylene

In experiments to trap an electrophilic intermediate of the 4-hydroxylation of cinnamic acid (Sandermann et al., 1977; Diesperger and Sandermann, 1978), the microsomal fraction from parsley cell suspension cultures was found to catalyze the addition of GSH to the olefinic double bond of cinnamic acid (Fig. 1A). In contrast to these results, the liver GSH S-transferases occur only as soluble enzymes (Boyland and Chasseaud, 1969; Jakoby, 1978) which are unable to add GSH to cinnamic acid (Boyland and Chasseaud, 1967).

The present communication deals with the occurrence of soluble and microsomal GSH S-cinnamoyl transferase activities in pea seedlings. A comparison of the fluorodifen-cleaving enzyme of pea seedlings (Frear and Swanson, 1973; Fig. 1B) and the formation of a GSH-conjugate of the carcinogen benzo[α]-pyrene is also reported.

Materials and Methods

Materials

Seeds of *Pisum sativum* L., var. Alaska, were kindly donated by Prof. R. Hertel of this Department. [3-^{14}C]Cinnamic acid and [7, 10-^{14}C]benzo[α]pyrene were purchased from CEA, Gif-sur-Yvette, France, and Amersham-Buchler, Braunschweig, respectively. [^{14}C-CF_3]Fluorodifen was kindly donated by Ciba-Geigy A.G., Basel. [3-^{14}C]p-Coumaric acid was prepared from [3-^{14}C]cinnamic acid using a parsley microsomal cinnamic acid 4-hydroxylase preparation (Diesperger et al., 1974). [3-^{14}C]Dihydrocinnamic acid was purchased from ICN Company, Irvine California. Soybean cell suspension cultures were grown as previously described (v.d. Trenck and Sandermann, 1978). Aged potato slices were prepared as described (Rich and Lamb, 1977).

General Procedures. The amounts of radioactivity and of protein were determined according to (Diesperger et al., 1974, Diesperger and Sandermann, 1978) and (Schaffner and Weissmann, 1973), respectively. Solvent systems for descending paper chromatography (Diesperger and Sandermann, 1978) were; (A) butanol-1/acetic acid/water, 2/1/1, by vol; and (B) benzene/acetic acid/water, 2/2/1,

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by vol, upper phase. The solvent system used for thin-layer chromatography on silica gel G (Frear and Swanson, 1973) was; (C) butanol-1/acetic acid/water, 12/3/5, by vol.

Enzyme Preparation. Pea seeds were soaked overnight in tap water and were then germinated in the dark as described by Russell (1971). After 12 days, primary leaves and 10 cm epicotyl sections were collected separately and homogenized at 4° C in 1 volume (per weight) of 50 mM Tricine × KOH, pH 7.5, by means of a household mixer (Krups model 3 Mix 3,000). The supernatant of a preliminary centrifugation (20 min, 10,000 *g*) was subjected to ultracentrifugation (90 min, 100,000 *g*). The microsomal pellets were suspended in small volumes of 50 mM Tricine × KOH, pH 7.5, and could be stored for several weeks at −20° C without loss of GSH S-cinnamoyl transferase activity. The supernatants from ultracentrifugation could also be stored for several weeks at −20° C. They were concentrated by ultrafiltration in an Amicon cell (UM-10 filter, $3.4 \cdot 10^2$ kN m^{-2}, 5° C) in order to remove most of an endogeneous transferase inhibitor. In most experiments (all of the kinetic experiments reported), the soluble enzymes were further purified by chromatography on a column of Sephacryl S-200 (cf. Fig. 4).

Enzyme Assays

General Procedure. All solutions used to determine enzyme activities contained 50 mM Tricine × KOH, pH 7.5. The standard incubations were carried out for 60 min at 30° C and were terminated by the application of the incubation mixture to chromatography paper, followed by development in the solvent system (A). Control incubations with heat-denatured enzyme (5 min, 95° C) were always run in parallel, and the control values of radioactivity were subtracted from the values obtained with the non-denatured enzyme. The assay mixtures were prepared by mixing the following ingredients.

GSH S-Cinnamoyl Transferase Assay. Enzyme solution (50 µl; containing 100–300 µg protein), 1.2 mM [3-^{14}C]cinnamic acid (50 µl; $3.8 \cdot 10^3$ d s^{-1}), 50 mM Tricine × KOH (20 µl) or, where indicated, 6 mM NADPH (20 µl), 125 mM GSH (5 µl) or, where indicated, 75 mM 2-mercaptoethanol (5 µl).

Assay for Cleavage of Fluorodifen (cf. Frear and Swanson, 1973). Enzyme solution (50 µl; containing appr. 100 µg protein), 176 mM [^{14}C-CF_3]fluorodifen (1 µl; $7.6 \cdot 10^2$ d s^{-1}), 6 mM GSH (50 µl).

Assay for GSH-Conjugation of Benzo[α]pyrene. Microsomal suspension (50 µl; containing 200–300 µg protein), 0.2 mM [7, 10-[^{14}C]benzo[α]pyrene (50 µl; $1.9 \cdot 10^3$ d s^{-1}; dispersed in 0.3% (v/v) Triton X-100, 0.3% (v/v) ethyleneglycolmonomethylether), 125 mM GSH (5 µl).

Results and Discussion

Nature of Products Formed

The GSH S-cinnamoyl adducts formed by the soluble and microsomal fractions of pea epicotyl and primary leaves (see below) were isolated by paper chromatography in solvent system (A). The previous chemical methods (Diesperger and Sandermann, 1978), in particular the release of dihydrocinnamic acid upon desulfurization by Raney nickel, indicated that in all cases GSH was linked to cinnamic acid by addition to the olefinic double bond, as shown in Fig. 1 A.

The product formed from the herbicide fluorodifen depended on the pH-value of the incubation mixture (Fig. 2). At pH 9.0; a product with Rf-values of 0.5 [solvent system (A)] and 0.33 [solvent system (C)] was formed. This product was probably identical with the intact GSH-adduct of Figure 1 B (cf. Frear and Swanson, 1973). However, a faster migrating product with Rf-values of 0.7 [solvent system (A)] and 0.6 [solvent system (C)] was formed at pH 7.5. At an intermediate pH-value of 8.25, both products were detected (Fig. 2).

These results appeared to indicate that the soluble enzyme fraction from pea seedlings contained some peptidase for a cleavage of the intact GSH-adduct of Fig. 1 B at pH-values below 9.0. No cleavage of the GSH S-cinnamoyl adduct of Fig. 1 A could be observed in the same soluble enzyme fractions at pH 7.5.

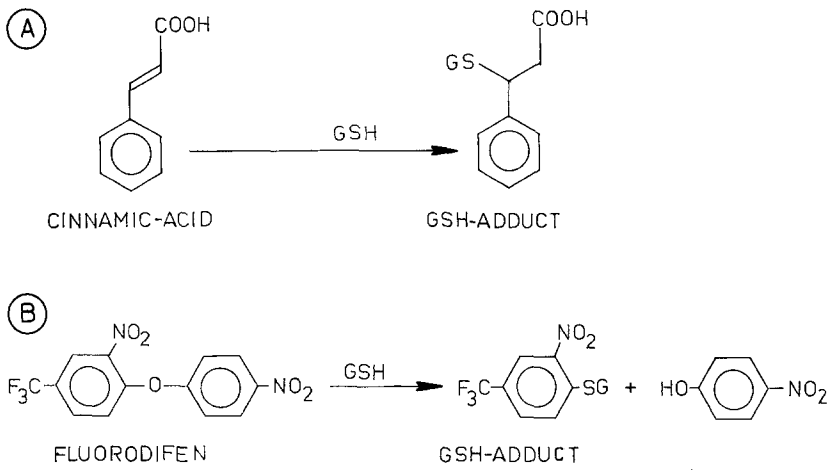

Fig. 1 A and B. Glutathione-dependent reactions catalyzed by enzymes from pea seedlings.
A Conjugation with cinnamic acid. The chemical characterization of the GSH S-cinnamoyl adduct did not differentiate between a thioether linkage to either C_2 or C_3 of cinnamic acid.
B Cleavage of the herbicide, fluorodifen (Frear and Swanson, 1973)

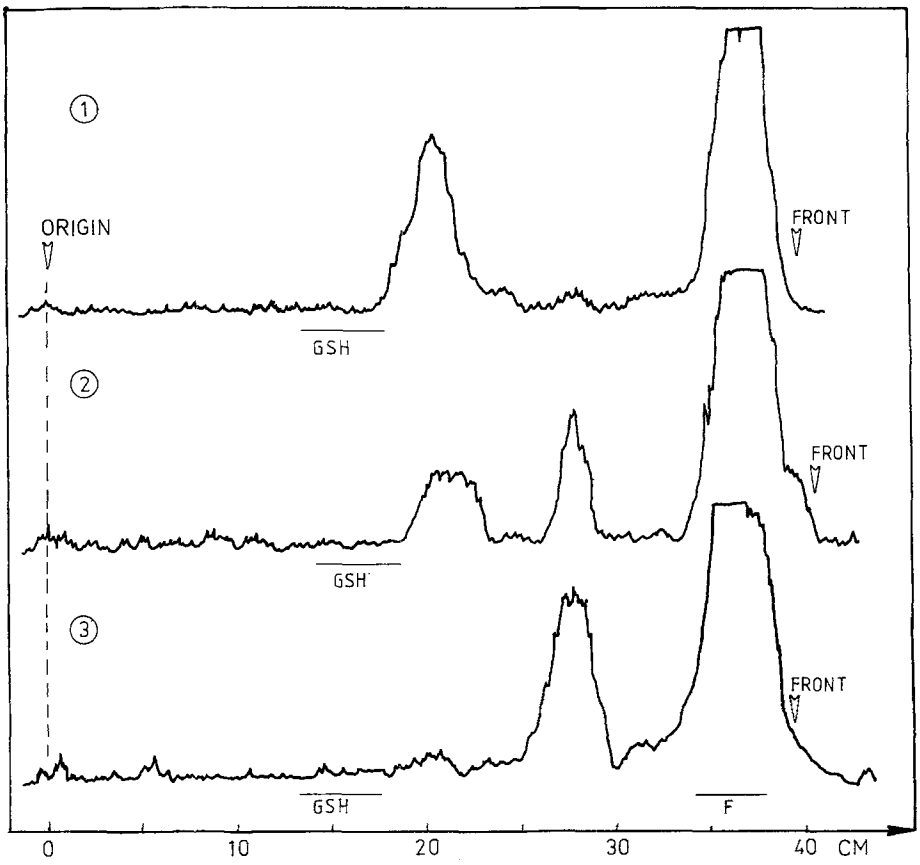

Fig. 2. Reaction between [^{14}C-CF_3]fluorodifen and glutathione as catalyzed by the purified soluble enzyme fraction from pea primary leaves. The figure shows the distributions of radioactivity on paper chromatograms [solvent system (A)] of incubations performed at 1: pH 9.0, 2: pH 8.25 and 3: pH 7.5. Abbreviations: GSH = glutathione; F = fluorodifen

Previous studies with whole peanut plants have indicated that the intact GSH-adduct of Fig. 1 B was processed to the S-cysteinyl adduct[1] (Shimabukuro et al., 1978).

Kinetic Properties of the GSH S-Cinnamoyl Transferase Activities from Pea Seedlings

The partially purified soluble enzymes and the microsomal activities had distinct pH-optima between pH 7.4 and 7.8, whereas, the non-enzymatic conjugation of GSH and cinnamic acid (cf. Diesperger and Sandermann, 1978; about 50 pmol adduct/min in the standard assay at pH 7.5) increased with increasing pH-values. The various transferase activities were linearly dependent on the amount of protein used (at least up to 300 µg protein per assay), as well as on the incubation time (up to 60 min). The purified, soluble and microsomal transferase activities were abolished by heat-denaturation (5 min, 95° C). In the case of the crude soluble extract from primary leaves, heating for 2 min at 95° C resulted in a 2–8-fold stimulation, and transferase activity was only abolished after 20 min at 95° C. Michaelis constants were determined by the Lineweaver-Burk procedure and were, in all cases, between 0.1–0.4 mM for cinnamic acid (in the presence of 5 mM GSH) and between 1–4 mM for GSH (in the presence of 0.5 mM cinnamic acid). V was 10–15 nmol adduct/mg protein × min for the purified soluble enzymes and 2–4 nmol adduct/mg protein × min for the microsomal fractions. The microsomal transferase activity remained associated with the microsomal fraction after repeated washing and after centrifugation through a 5 cm cushion of 1 M glucose, 100 mM Tris-HCl, pH 7.5 (90 min, 100,000 *g*).

[1] The second split product of fluorodifen cleavage, p-nitrophenol (cf. Fig. 1 B), is further processed in peanut to the 6-O-malonyl-β-D-glucopyranosyl-derivative (Frear, 1976). A cell-free malonyl-transfer from malonyl-SCoA to β-D-glucopyranosyl-p-nitrophenolate has recently been demonstrated with a highly purified malonyl-transferase from parsley cell suspension cultures (U. Matern and H. Sandermann, unpublished results)

GSH S-Cinnamoyl Transfer in Other Plants

The microsomal fraction from parsley cell suspension cultures (Diesperger and Sandermann, 1978) had V, 1 nmol adduct/mg protein × min. A microsomal frac-

tion from potato slices (Rich and Lamb, 1977) had V, about 10 pmol adduct/mg protein × min.

When tested in the presence of 1 mM NADPH, the potato microsomal fraction formed the Rf-0.4 GSH-adduct of cinnamic acid [solvent system (A)] which was probably derived from cinnamic acid 3.4-epoxide (Diesperger and Sandermann, 1978). In addition, smaller amounts of products which co-migrated with p-coumaric and caffeic acid [solvent systems (B)] and a cinnamoyl-compound remaining at the origin in the solvent system (B), were formed. These various products were not formed by heat-denatured enzyme (5 min, 95° C) and required the presence of NADPH. Addition of the soluble protein fraction of potato slices led to the previously reported increase in p-coumaric acid formation (Rich and Lamb, 1977) at the expense of the cinnamoyl-compound at the origin.

No GSH S-cinnamoyl transferase activity was detected in the crude soluble enzyme fraction from potato slices (Rich and Lamb, 1977) or from parsley cell suspension cultures (Diesperger and Sandermann, 1978). It has not been determined whether this was caused by the presence of an endogeneous inhibitor which was present in the crude soluble extract from the pea seedlings (data not shown).

Soybean cell suspension cultures were found to contain soluble and microsomal GSH S-cinnamoyl transferase activities with V, 2 nmol adduct/mg protein × min for the microsomal fraction, and V, 4.5 nmol adduct/mg protein × min for the crude soluble fraction.

Intact log-phase soybean cell suspension cultures (40 ml) were incubated for 2 h at 25° C with [3-^{14}C]-cinnamic acid (0.5 mM; $3.8 \cdot 10^4$ d s^{-1}) without the addition of GSH. A 6% conversion to the GSH S-cinnamoyl adduct of Fig. 1 A was found upon paper chromatography in the solvent system (A), and about equal amounts of the adduct were isolated from the cells and from the growth medium. A 1.4% conversion was found in the autoclaved control cultures (cf. v. d. Trenck and Sandermann, 1978).

Alternative Substrates

No detailed studies on alternative transferase substrates have been carried out. This section is, therefore, limited to some qualitative results obtained with the pea microsomal fractions. A GSH-adduct was formed when [3-^{14}C]p-coumaric acid was employed instead of [3-^{14}C]cinnamic acid, but [3-^{14}C]dihydrocinnamic acid did not serve as a substrate. 2-Mercaptoethanol was able to partially replace GSH, and the adduct formed with cinnamic acid had an Rf-value of 0.25–0.4 in the solvent system (B). p-Coumaric acid has the same Rf-value in this solvent system which is in use for the assay of microsomal cinnamic acid 4-hydroxylase activity (Russell, 1971). Furthermore, 2-mercaptoethanol has been reported to be a strong activator of the pea cinnamic acid 4-hydroxylase (Russell, 1971). The present results indicate that the simple addition of 2-mercaptoethanol to cinnamic acid is also catalyzed by the pea microsomal fraction, and that this reaction may interfere with the chromatographic determination of p-coumaric acid.

The microsomal fraction from pea primary leaves also catalyzed the formation of a GSH-adduct with the carcinogen benzo[α]pyrene, leading to a product with an Rf-value of 0.55 in the solvent system (A) [V, 30 pmol adduct/mg protein × h). This product was not formed in the absence of GSH or with heat-denatured enzyme (5 min, 95° C). Subsequent results indicated that the microsomal fraction from pea primary leaves also catalyzes the formation of oxygenated metabolites of benzo[α]pyrene so that the GSH-adduct may be formed as a secondary product (T. v. d. Trenck and H. Sandermann, unpublished results).

Inhibitors

The following compounds gave less than 15% inhibition of microsomal GSH S-cinnamoyl transfer when added at 5 mM to the standard assay mixture; allyl alcohol, propachlor, styrene oxide, aldrin, dieldrin, endrin, DDE, DDMU, heptachlor, heptachlorepoxide, dihydrocinnamic acid, and atrazine. These inhibition effects were corrected for the appr. 25% inhibition given by the organic solvents used at final concentrations of 4%, v/v (methanol, dimethylsulfoxide or ethyleneglycol monomethylether). The addition of 5 mM styrene resulted in 27% inhibition; 5 mM cinnamyl alcohol inhibited by 76%, and 5 mM 3.4-methylenedioxy-cinnamic acid gave complete inhibition. 2-Mercaptoethanol, which was accepted as an alternative substrate (see above), was a strong inhibitor of the soluble and microsomal GSH S-cinnamoyl transfer reactions (50% inhibition at 0.5–2 mM 2-mercaptoethanol). Lineweaver-Burk plots (not shown) indicated a non-competitive type of inhibition.

Apparent Molecular Weights

The soluble enzyme fractions were chromatographed on a column of Sephacryl S-200, and similar results were obtained for the extracts from epicotyl and primary leaves. In the experiment shown in Fig. 3, the soluble GSH S-cinnamoyl transferase activity appeared in two molecular weight regions corresponding to 37,000 and 150,000, respectively. In other experiments an additional peak corresponding to 75,000

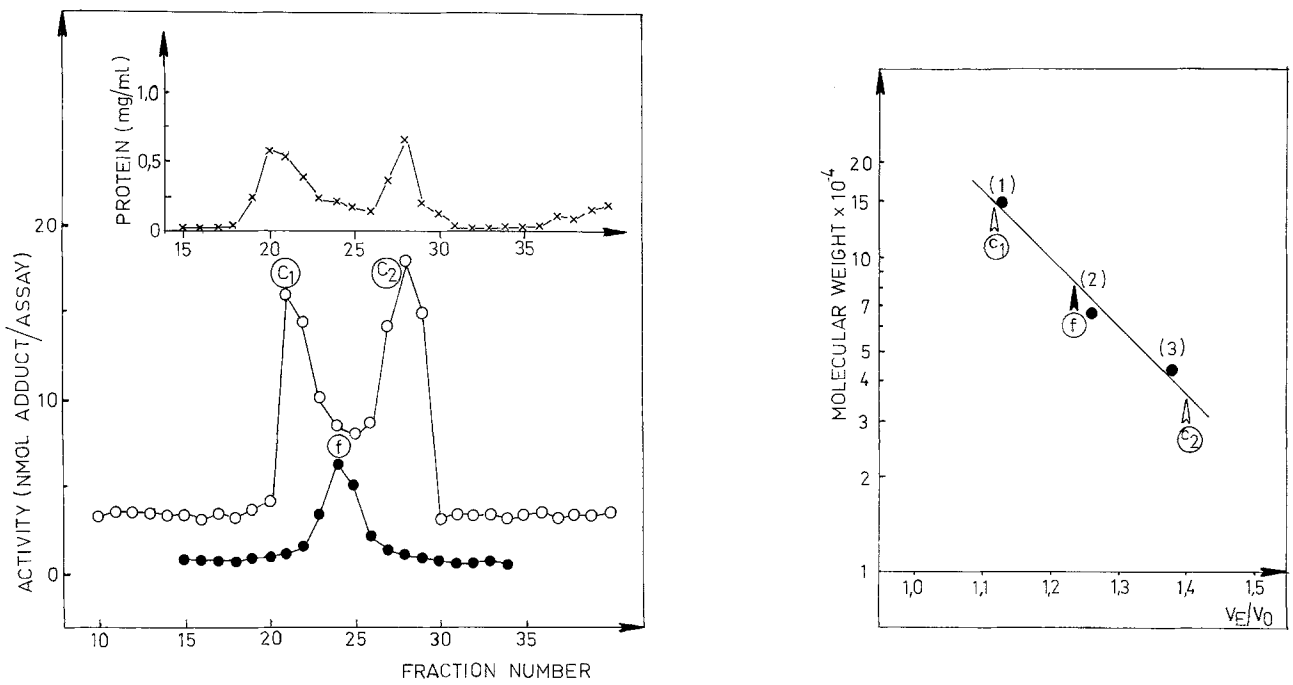

Fig. 3. Chromatography of the soluble enzyme preparation from pea primary leaves on a column (90 × 1.5 cm) of Sephacryl S-200 in 50 mM Tricine × KOH, pH 7.5. The sample (76 mg protein) was applied in 2 ml 50 mM Tricine × KOH, pH 7.5, and fractions of about 3.1 ml were collected every 30 min. Left panel. The distribution of protein (×—×) is shown in the upper part of the graph. The lower curves show the distributions of GSH S-cinnamoyl transfer (o—o) and of fluorodifen-cleavage (●—●), as determined with 50 µl aliquots of each fraction. Right panel. Calibration curve for the column used. The following standard proteins were employed, 1. aldolase, 2. bovine serum albumin, 3. egg albumin. The elution position of blue dextrane was taken as V_0. The peak positions of GSH S-cinnamoyl transfer and of fluorodifen-cleavage are shown as C_1 and C_2 and f, respectively

was observed. When the 150,000 species was re-chromatographed on Sephacryl S-200, it gave rise to the 37,000 enzyme species. The GSH S-cinnamoyl transferase activity thus appeared to be associated with a protein of molecular a weight of 37,000, which had a tendency to aggregate to dimeric and tetrameric forms.

The fluorodifen-cleaving enzyme activity appeared at a molecular weight of appr. 82,000 (Fig. 3). In other experiments, activity also appeared at a molecular weight of appr. 47,000. The enzyme reactions of Figure 1 A and Figure 1 B therefore appeared to be catalyzed by different enzymes.

Possible Physiological Role of GSH-Conjugation

The physiological function of GSH S-cinnamoyl adducts is obscure at present. They may be transport or storage forms but it cannot be excluded that the above GSH S-transferases may really catalyze another reaction, such as the addition of cysteine residues to the olefinic double bonds of the chromophores of phytochrome (Klein et al., 1977) or cytochrome c (Conn and Stumpf, 1976). The formation of GSH-adducts of the carcinogen benzo[α]pyrene, the herbicide fluorodifen, as well as a number of additional herbicides (Shimabukuro et al., 1978) indicates that conjugation reactions with glutathione may constitute an important pathway for the detoxification of xenobiotics by plants.

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